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Fluoro Ketone Inhibitors of Hydrolytic Enzymes†

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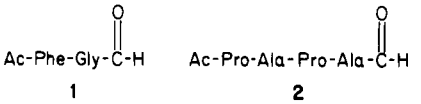
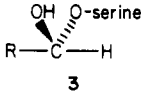
ABSTRACT: The use of fluoro ketones as inhibitors of hydrolytic enzymes has been investigated. The acetylcholine analogues 6,6-dimethyl-1,1,1-trifluoro-2-heptanone and 3,3-difluoro-6,6-dimethyl-2-heptanone are inhibitors of acetylcholinesterase with K_i values of 16×10^{-9} M and 1.6×10^{-9} M, respectively. These fluoro ketones are 10^4–10^5 times better as inhibitors than the corresponding methyl ketone. Since nucleophiles readily add to fluoro ketones, it is likely that these compounds inhibit acetylcholinesterase by formation of a stable hemiketal with the active-site serine residue. Fluoro ketone substrate analogues are also inhibitors of zinc metallo- and aspartylproteases. 2-Benzyl-4-oxo-5,5,5-trifluoropentanoic acid is an inhibitor of carboxypeptidase A ($K_i = 2 \times 10^{-7}$ M). Trifluoromethyl ketone dipeptide analogues are good inhibitors of angiotensin converting enzyme. An analogue of pepstatin that contains a difluorostatone residue in place of statine has been prepared and found to be an extremely potent inhibitor of pepsin ($K_i = 6 \times 10^{-11}$ M). The hydrated ketones are probably the inhibitory species since they are structural mimics of the tetrahedral intermediate that forms during the hydrolysis of peptide substrates.

Peptides and amino acid analogues containing aldehydic functional groups are good inhibitors of serine and cysteine proteases. For example, papain is inhibited by **1** ($K_i = 5 \times 10^{-8}$ M) (Westerik & Wolfenden, 1971) and elastase is inhibited by **2** ($K_i = 8 \times 10^{-7}$ M) (Thompson, 1973). Since



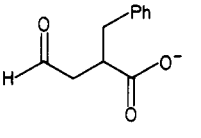
1 **2**

aldehydes are largely hydrated in aqueous solution, it is likely that the aldehyde in the active site exists bound to the serine oxygen as a hemiacetal (**3**). Evidence for a bound hemiacetal



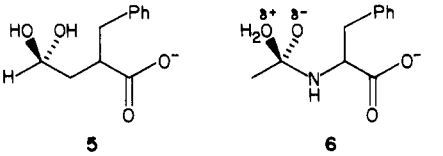
3

has been provided by NMR spectroscopy (Lowe & Nurse, 1977; Chen et al., 1979; Shah et al., 1984). Since the structure of the enzyme-bound hemiacetal resembles the tetrahedral intermediate that is formed during the hydrolysis of peptide substrates, it has been suggested that aldehydic peptides are transition-state analogue inhibitors (Wolfenden, 1976). Aldehydes are also inhibitors of metalloproteases. For example, **4** is an inhibitor of carboxypeptidase A (Galardy & Kortylewicz, 1984). The hydrated form **5**, which resembles the



4

tetrahedral adduct **6** formed when H_2O adds to the carbonyl group of a peptide substrate, is probably the inhibitory species.



5 **6**

Aldehydic peptides, although efficient inhibitors, have certain limitations. Aldehydes can only be placed on the carboxyl-terminal side of peptides. Since proteases interact

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with groups on both sides of the scissile peptide bond (Fruton, 1976), protease inhibitors that make use of these long-range interactions are likely to have a higher degree of specificity toward targeted proteases. Peptide derivatives that contain recognition structures on both sides of an internal ketone can be prepared. However, these compounds are not likely to be good inhibitors of proteases because of the low tendency of ketones to hydrate in solution or to form a hemiketal with an active-site nucleophile. Introduction of fluorine on a carbon adjacent to the carbonyl group will enhance the tendency of the ketones to add nucleophiles, and thus peptides containing fluoro ketones should be good inhibitors of proteases. Both di- and trifluoromethyl ketones exist in water almost entirely in the hydrated form, and thus it seemed likely that fluoro ketones would form a hemiketal at the active site of a serine protease or, as hydrates, bind tightly to both metallo- and aspartylproteases. It has been shown that *m*-(trimethylammonio)trifluoroacetophenone inhibits acetylcholinesterase (Brodbeck et al., 1979). Inhibition is probably the result of formation of a stable hemiketal with the active-site serine residue. Juvenile hormone esterase is also inhibited by alkyl trifluoromethyl ketones ($K_i = 3.2 \times 10^{-9}$ M) (Hammoch et al., 1982; Prestwich et al., 1984). We have synthesized a number of di- and trifluoromethyl ketones and have examined their inhibitory properties toward several proteases as well as acetylcholinesterase.

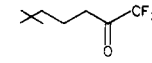
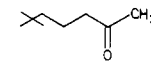
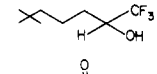
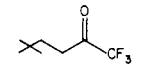
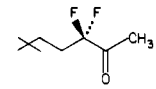
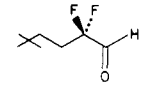
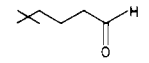
Experimental Procedures

Acetylcholinesterase (1400 units/mg) from electric eel (type V-S) was from Sigma. It was assayed in 100 mM potassium phosphate, pH 7.0, with acetylthiocholine (Aldrich) (Ellman et al., 1961). Carboxypeptidase A from bovine pancreas (type I) was purchased from Sigma and assayed in 50 mM 4-morpholinepropanesulfonic acid, pH 7.5, and 0.5 M NaCl with hippuryl-DL-phenyllactic acid (Sigma) (Folk & Schirmer, 1963). A preparation of rabbit lung angiotensin converting enzyme suitable for testing inhibitors was prepared as described (Bush et al., 1984) and assayed in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, and 0.3 M NaCl with *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (Sigma) as substrate (Holmquist et al., 1979). Bovine pepsin was from Worthington. Pepsin assays were performed in 40 mM formate, pH 4.0, by monitoring the decrease in absorbance at 300 nm accompanying the hydrolysis of a heptapeptide substrate. The heptapeptide substrate Lys-Pro-Ala-Glu-Phe(NO_2)-Arg-Leu ($K_m = 50$ μM, $k_{cat} = 100$ s^{-1}) was a generous gift from Professors Paul Bartlett, University of California, Berkeley, and Ben Dunn, University of Florida. Equivalent pepsin assays were also performed with Phe-Gly-His-Phe(NO_2)-Phe-Val-Leu-OMe, obtained as a generous gift from Professor Joseph Fruton, Yale University.

All spectrophotometric enzyme assays were performed on a Perkin-Elmer λ-3 UV/vis spectrophotometer using 1-cm quartz cells thermostated at 25 °C. All of the inhibitors in this study were reversible competitive inhibitors, and K_i values were determined from Lineweaver–Burk plots using the initial velocities measured from the linear portion of the absorbance vs. time reaction-progress curves. In the case of inhibition of pepsin by **26**, less than 100% inhibition occurred only with inhibitor concentrations approaching the concentration of enzyme. The value of K_i was estimated by using the steady-state equation for competitive inhibition that accounts for the significant depletion of free inhibitor that results from binding to the enzyme (Williams & Morrison, 1979).

A detailed description of the syntheses of all new compounds and their physical properties is presented in the supplementary material (see paragraph at end of paper regarding supplementary material).

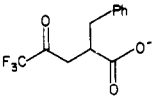
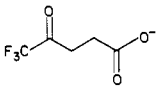
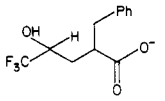
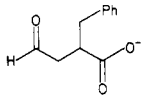
Table I: Inhibitors of Acetylcholinesterase

no.	compound	K_i (nM)
7		16
8		310 000
9		410 000
10		8 000
11		1.6
12		600
13		$>10^6$

Results and Discussion

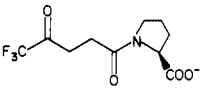
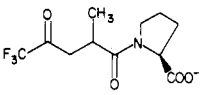
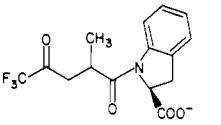
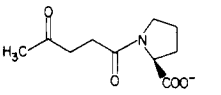
Acetylcholinesterase. We synthesized fluoro ketone analogues of acetylcholine as potential inhibitors of acetylcholinesterase. To simplify the synthetic problems, we initially prepared trifluoromethyl ketones without the trimethylammonium group. The results obtained are shown in Table I. The trifluoromethyl ketone **7** is an effective, reversible inhibitor of acetylcholinesterase. The K_i is (2×10^4)-fold lower than the K_i of the corresponding methyl ketone **8** or that of the corresponding trifluoromethyl alcohol **9**. The trifluoromethyl ketone **10**, which is shorter than **7** by a single methylene group, is a much poorer inhibitor. Surprisingly, the K_i for the difluoromethyl ketone **11** is 10-fold lower than that of the trifluoromethyl ketone **7**. However, these ketones are not strictly comparable since the fluorine atoms are not in corresponding positions. Possibly the trifluoromethyl group interferes with binding of the inhibitor. The rate of dissociation of the enzyme bound to **7** was measured by diluting the inactivated enzyme into a large excess of buffer containing the assay substrate. Enzymatic activity returned in a first-order manner, giving a value for k_{off} of 2.5×10^{-3} s^{-1}. The association rate constant can be calculated from the expression $k_{on} = k_{off}/K_i$ and has a value of 1.6×10^5 M^{-1} s^{-1}. The difluoro aldehyde **12** is a poorer inhibitor than the corresponding ketones **7** and **11**, although its K_i is 10^3–10^4 lower than that of the corresponding aldehyde **13**. Of the compounds listed in Table I, time-dependent inhibition is seen only with **12**. The dissociation rate constant of the enzyme bound to difluoro aldehyde **12** was measured by separating the inactivated enzyme from excess inhibitor by rapid gel filtration (Penefsky, 1979). The return of enzymatic activity was followed by periodically assaying portions of the mixture by dilution into buffer containing the assay substrate. Activity returned in a first-order fashion, giving a value for k_{off} of 1.8×10^{-3} s^{-1}. The association rate constant ($k_{on} = k_{off}/K_i$) is calculated to be 3×10^3 M^{-1} s^{-1}. This association rate constant for **12** is 50-fold smaller than for the trifluoromethyl ketone **7**. Possibly, in all cases, the unhydrated form of the inhibitor reacts with the enzyme. The concentration of unhydrated aldehyde is probably considerably lower than that of unhydrated ketone [the hydration equilibrium constant for trifluoroacetaldehyde is

Table II: Inhibitors of Carboxypeptidase A

no.	compound	K_i (μM)
14		0.2
17		700
18		90
4		0.48[a]

[a] Galardy & Kortylewicz, 1984.

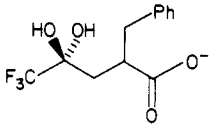
Table III: Inhibitors of Angiotensin Converting Enzyme

no.	compound	K_i (μM)
19		15
20		0.2
21		0.012
22		4500[a]

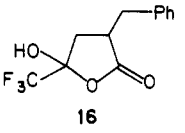
[a] Petrillo & Ondetti, 1982.

estimated to be greater than 10^4 compared to a value of 35 for trifluoroacetone (Guthrie, 1975)], and therefore, the dehydration may be partially rate determining in the reaction between aldehyde and enzyme. We assume, although this point has not been established, that, in all cases, inhibition is due to the formation of a tetrahedral adduct between the fluoro aldehydes or fluoro ketones and the active-site serine of acetylcholinesterase.

Inhibitors of Zinc Metalloproteases. Carboxypeptidase A is weakly inhibited by ketomethylene substrate analogues. The phenylalanine analogue (−)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid reversibly inhibits carboxypeptidase A with a K_i of 100 μM (Sugimoto & Kaiser, 1978). We anticipated that the placement of fluorine on a carbon atom adjacent to the carbonyl group would increase the potency of inhibition by ketomethylene analogues. We prepared DL-2-benzyl-4-oxo-5,5,5-trifluoropentanoic acid (**14**) as a possible inhibitor of carboxypeptidase A. The trifluoromethyl ketone **14** exists in aqueous solution near neutrality in the monoanionic, hydrated form **15**. The free acid cyclizes to the cyclic hemiacetal **16** in organic solvents.



15



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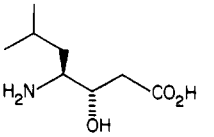
Compound **14** is a potent inhibitor of carboxypeptidase A (K_i = 0.2 μM; Table II). The K_i of the corresponding aldehyde **4** (Table II) is approximately 2-fold higher (Galardy & Kortylewicz, 1984). The unsubstituted trifluoropentanoic acid **17** that lacks the 2-benzyl group is a much poorer inhibitor of carboxypeptidase A (Table II). The K_i for **14** is 450-fold smaller than the K_i for the trifluoromethyl alcohol **18**. It is likely that the strong inhibition is the result of the formation of a tightly bound tetrahedal adduct that resembles the tetrahedral intermediate that forms during the hydrolysis of substrates. Whether this tetrahedral adduct is the hydrated ketone or is formed by the addition of Glu-270 to the trifluoromethyl ketone is not known.

Angiotensin converting enzyme is a zinc metalloprotease that cleaves a dipeptide from the carboxyl terminus of angiotensin I to produce angiotensin II. Angiotensin II is a potent vasoconstrictor; therefore, inhibitors of the converting enzyme are likely candidates for antihypertension pharmaceuticals (Petrillo & Ondetti, 1982). Since its mechanism of action is very similar to that of carboxypeptidase, trifluoromethyl ketone containing dipeptide analogues should also inhibit angiotensin converting enzyme. Results are shown in Table III. Compounds **19** and **20** are analogues of the dipeptides glycylproline and alanylproline, respectively. Many angiotensin converting enzyme inhibitors have been modeled after these dipeptides (Petrillo & Ondetti, 1982). Both **19** and **20** are potent inhibitors of angiotensin converting enzyme. It is significant that the trifluoromethyl ketone **19** is 300-fold more potent than the methyl ketone **22**. This result demonstrates the importance of fluorine substitution adjacent to the ketone. Inhibitor **21** contains a benzo substituent fused to the proline ring and is the most potent compound in this series (Table III). This inhibitor was modeled after the dipeptide inhibitor alanyltryptophan (Cheung et al., 1980).

We believe that, by substituting additional recognition groups in place of one of the fluorine atoms of the trifluoromethyl group, much more effective inhibitors for carboxypeptidase as well as angiotensin converting enzymes can be obtained.

Inhibitors of Pepsin. We also explored the possibility of inhibiting aspartylproteases (i.e., pepsin and renin) with fluoro ketones. We synthesized a fluoro ketone based on pepstatin. Pepstatin is a natural product isolated from microbial sources and is found to be an extremely potent inhibitor of pepsin (K_i = 4.6 × 10^{-11}) (Umezawa et al., 1970; Workman & Burkitt, 1979). Pepstatin is a pentapeptide that contains an unusual amino acid called statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (**23**)]. Since statine is structurally

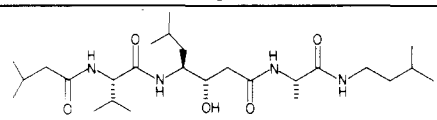
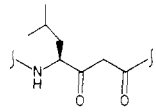
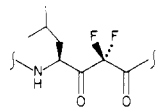
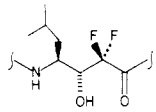


23

related to the tetrahedral intermediate formed during pepsin-catalyzed hydrolysis of peptides, it has been suggested that statine-containing peptides are transition-state analogue inhibitors of aspartylproteases (Marciniszyn et al., 1976).

Synthetic analogues of pepstatin have been prepared in order to define the structural features of pepstatin that are required for tight binding to pepsin (Rich et al., 1984). For example, compound **24** (Table IV) is a tight-binding inhibitor of pepsin (Rich et al., 1980). Oxidation of the secondary alcohol of statine to the ketone produces the statone-containing peptide

Table IV: Inhibitors of Pepsin

no.	compound	K_i (nM)
24		1.1[a]
25		56[b]
26		0.06
27		0.5

[a] Rich et al., 1980. [b] Rich et al., 1982a.

25 (Table IV). Compound **25** is an inhibitor of pepsin but is 50-fold less effective than the statine-containing peptide **24** (Rich et al., 1982a).

Interestingly, a recent NMR study employing carbon-13-enriched statone-containing peptides demonstrated that the ketone sp^2 carbon has undergone a transition to the sp^3 form when complexed to pepsin (Rich et al., 1982b). This result suggested that the inhibitor is bound to the enzyme in the hydrated form (Rich et al., 1984). The 50-fold decrease in binding to the enzyme might be attributed to the poor tendency of ketones to undergo a change to the tetrahedral form. In order to produce a more potent inhibitor, we reasoned that the substitution of fluorine in the methylene group of statone would facilitate the ease of hydration of the ketone. The difluorostatone-containing peptide **26** was synthesized. Carbon-13 NMR analysis of **26** in chloroform demonstrated that it exists in the β-ketoamide form. In dimethyl sulfoxide–water (4:1), the ketone is predominantly in the hydrated form. Inhibition results are presented in Table IV. The difluorostatone-containing peptide **26** was found to be an extremely potent inhibitor of pepsin ($K_i = 6 \times 10^{-11}$ M). In order to test whether **26** is a slow-binding inhibitor of pepsin, an experiment was carried out in which enzyme (5×10^{-9} M) and inhibitor **26** (7×10^{-9} M) were allowed to incubate for a variable amount of time before the reaction was initiated by the addition of substrate. Maximal inhibition was obtained in less than 1 min. From this experiment, k_{on} is calculated to have a value of at least 3×10^6 M^{-1} s^{-1}. Thus inhibitor **26** is not a slow-binding inhibitor of pepsin. The difluorostatine analogue **27** inhibits pepsin with a K_i of 0.5×10^{-9} M (Table IV). A similar K_i is reported for the nonfluorinated statine-containing peptide **24**. Compound **27** is not a slow-binding inhibitor.

The results presented above demonstrate that good inhibitors derived from fluoro ketones can be made for acetylcholinesterase as well as for metallo- and aspartylproteases. In all cases, the fluoro ketones are much more effective inhibitors than the corresponding nonfluorinated ketones.

The equilibrium between the unbound and enzyme-bound fluoro ketone inhibitor is

$$\text{E} + \text{hydrate} \overset{K_{i,F}}{\rightleftharpoons} \text{E·hydrate} \qquad (1)$$

$$K_{i,F} = [\text{hydrate}][\text{E}]/[\text{E·hydrate}]$$

E·hydrate refers to the enzyme-bound hemiketal, in the case of acetylcholinesterase, or to the enzyme-bound hydrated ketone, in the case of zinc metallo- and aspartylproteases. The free and bound ketone species have not been included in eq 1 because they are present at insignificant concentrations. The results presented in this paper demonstrate that the equilibrium in eq 1 lies far to the right ($K_{i,F} = 10^{-6}$–10^{-11} M). There is no change in the hydration state of the inhibitor when it binds to the enzyme, and thus the tight binding must be the result of the favorable interactions between the bound hemiketal or hydrate and the enzyme. The bound hemiketal or hydrate resembles the structure of the tetrahedral intermediate that is formed during the hydrolysis of a peptide substrate. It is, therefore, likely that the fluoro ketones are transition-state analogue inhibitors of proteolytic enzymes (Wolfenden, 1976).

The nonfluorinated ketones studied in this work are much poorer inhibitors than the corresponding fluoro ketones. The equilibrium between free and bound methyl ketone is

$$\text{E} + \text{hydrate} \overset{K_{2,H}}{\rightleftharpoons} \text{E·hydrate} \qquad (2)$$

$$\text{ketone} \overset{K_{1,H}}{\rightleftharpoons} \text{hydrate}$$

$$K_{2,H} = [\text{hydrate}][\text{E}]/[\text{E·hydrate}]$$

$$K_{1,H} = [\text{ketone}]/[\text{hydrate}]$$

In this case, since the major free inhibitor species is the ketone, the observed $K_{i,H}$ will be the product:

$$K_{i,H} = [\text{ketone}][\text{E}]/[\text{E·hydrate}] = K_{1,H}K_{2,H}$$

The ratio of K_i's for the fluorinated and nonfluorinated inhibitors is then

$$K_{i,F}/K_{i,H} = K_{i,F}/(K_{1,H}K_{2,H})$$

In order to estimate the value of this ratio, a comparison of the constants $K_{i,F}$ and $K_{2,H}$ needs to be made. Since the steps defined by $K_{i,F}$ and $K_{2,H}$ do not involve a change in the hydration state of the inhibitor, their values should be very similar and we assume that $K_{i,F} = K_{2,H}$. This assumption ignores the possibility that the bound hydrate might ionize on the enzyme. The pK_a values for the ionization of trifluoromethyl ketone hydrates to the monoanion are near 10 (Bell, 1966). Ionization could provide an additional favorable interaction between the bound inhibitor and the enzyme. This assumption also ignores any steric differences between the fluorinated and nonfluorinated ketones. Such effects are expected to be small. With this assumption, $K_{i,F}/K_{i,H}$ becomes $1/K_{1,H}$. The value of $K_{1,H}$ can be estimated from the reported hydration equilibrium for acetone of 10^3 (Hine & Redding, 1970). The above analysis predicts that the fluoro ketones should have K_i's that are ca. 10^3-fold smaller than the K_i's for the nonfluorinated ketones. This is observed experimentally. For pepsin, the K_i for the difluorostatone peptide **26** is (1.2×10^3)-fold smaller than for the statone peptide **25**. For acetylcholinesterase, the K_i for the trifluoromethyl ketone **7** is (2×10^4)-fold smaller than the K_i for the methyl ketone **8**. In this latter case, the larger than expected factor suggests that there is an additional advantage to binding the fluorinated inhibitor. As discussed above, it is possible that the bound trifluoromethyl hemiketal undergoes ionization and this further stabilizes the complex.

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SUPPLEMENTARY MATERIAL AVAILABLE

A detailed description of the syntheses of all new compounds and their physical properties (12 pages). Ordering information

is given on any current masthead page.

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Articles

Evidence for Active Intermediates during the Reconstitution of Yeast Phosphoglycerate Mutase

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ABSTRACT: The reconstitution of the tetrameric phosphoglycerate mutase from bakers' yeast after denaturation in guanidine hydrochloride has been studied. When assays are performed in the presence of trypsin, it is found that reactivation parallels the regain of tetrameric structure. However, in the absence of trypsin, the regain of activity is more rapid, suggesting that monomeric and dimeric intermediates possess partial activity (35% of the value of native enzyme) which is sensitive to trypsin. When reconstitution is studied in the presence of substrates, it is again found that monomeric and dimeric intermediates possess 35% activity. Under these latter conditions, the activity of the monomer but not of the dimer is sensitive to trypsin.

The reconstitution of oligomeric enzymes after denaturation has become a useful experimental model for the folding and association of these enzymes during biosynthesis [for a review, see Jaenicke (1984)]. Comparative studies of the rates of regain of enzyme activity and of quaternary structure, e.g., by use of the glutaraldehyde cross-linking technique (Hermann et al., 1981), have allowed the kinetic aspects of the pathway of reconstitution to be explored for a number of enzymes and permitted deductions to be drawn regarding the catalytic properties of intermediate species. In most cases, it appears

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